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TITLE: DEVELOPMENT OF AN AQUATIC BIOASSAY USING THE MEDAKA (ORYZIAS LATIPES) TO ASSESS HUMAN HEALTH RISK: TUMOR IMMUNODIAGNOSIS

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In a continuation project for the development of aquatic bioassays as alternatives for carcinogenicity and toxicity testing to assess human health risk, immunohistochemical techniques (IHC) for the diagnosis of carcinogen induced neoplasms and other proliferative lesions in fish will be developed. Groups of medaka (Oryzias latipes) have been exposed to either N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or methylazoxymethanol acetate (MAN-Ac) to induce variable neoplasms and proliferative lesions to which IHC can be applied to identify differentiation antigens. Both peroxidase-antiperoxidase and avidin biotin complex techniques are used to identify intermediate filament proteins keratin, desmin, vimentin, glial fibrillary acidic protein, and neurofilament protein. Other selected antigens may also be tested for based on histology. These findings will be correlated with histology, and ultrastructure. The results of this proposal will identify association between cell types in neoplasms and in proliferative lesions of uncertain origin which cannot yet be types by morphology alone in the fish, and thereby identify the possible progression of these lesions to neoplasms. These data are essential to the development of aquatic bioassays to evaluate human health risk and thus this research will contribute significantly to the development of the model.

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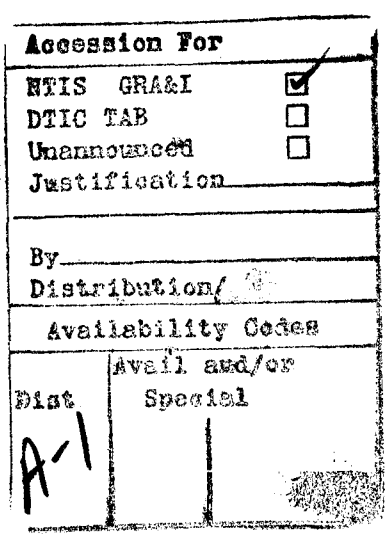

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INTRODUCTION

The use of fish species as alternative animal models in carcinogenicity and toxicity testing has been validated over the last several years through the efforts of numerous investigators. This has been made possible through support from the Research Methods Branch of the Health Effects Research Division at the United States Army Biomedical Research and Development Laboratory. The data generated have wide ranging implications from environmental and human health perspectives. The ability to recognize the deleterious effects of contaminants on fish populations enhances problem solving abilities associated with contaminated environmental sites and in the establishment of human health risk. Pathology has had an integral role in this process.

Originally, it was thought that aquatic bioassays needed to mimic rodent carcinogenicity bioassays to be valid. However, it was determined that the assessment of risk is based more on whether a compound produces a lesion which 1) is permanent, 2) progresses to cancer, 3) is predictable (reproducible), and 4) is deleterious to the health of the species.

In the original contract (DAMD17-88-C-8029, USABRDL), four groups of 14-day old medaka were exposed for 48 hours to 0, 100, 200, or 400 mg/L of diethylnitrosamine (DEN) and sequentially sacrificed at approximately 2, 4, 6, 8, 12, and 24 weeks post exposure to determine the effects of short term exposure protocols (DAMD17-88-C-8029) (4,5). Most of the lesions seen were similar to those reported in bioassays using adults of various species of fish exposed to DEN, including foci of cellular alteration, cellular vacuolation, eosinophilic inclusions, and a variety of benign and malignant hepatic neoplasms. Both the incidence and severity of lesions induced, including

malignancy, were directly related to exposure level (Table 1) (5). In another study with USABRDL, medaka embryos (2 days prehatching age) were exposed to methylazoxymethanol acetate (MAM-Ac) for 4 hours at 20, 25, or 30°C followed by a grow-out period of up to one year. Numerous neoplasms developed in several organs although muscle tumors and other sarcomas seemed to predominate (Tables 2,3).

However, in both the DEN and MAM-Ac studies at USABRDL, unusual proliferative lesions were seen in liver and soft tissues which were difficult to identify by morphologic criteria alone, and which are not features of rodent bioassays. In addition, many neoplasms in the two studies were tentatively diagnosed (see *, Tables 1 and 2) because morphology alone was insufficient, and because of limited data on the origin and behavioral biology of certain cell types in the fish.

Table 1. Total benign and malignant hepatic neoplasms in medaka exposed to DEN (original contract #DAMD17-88-C-8029) (5)

Type	Exposure group and total number of each tumor type
Adenoma	II (1), III (1)
Cholangioma	II (1)
Hepatocellular carcinoma	I (1), II (2)
Cystadenocarcinoma	II (1)
Cholangiocarcinoma	II (1), III (1)
*Sarcoma with vascular orientation	II (1), III (2)
*Histiocytic sarcoma	II (1), III (1)
*Carcinosarcoma	III (1)
*Malignant - undetermined type	II (1), III (1)

*Tentative based on histology and ultrastructure
Group I = 100 mg/L DEN; Group II = 200 mg/L DEN; Group III = 400 mg/L DEN.

Table 2. Number of neoplasms seen per total number of fish taken at each sacrifice for each group. Number of malignant neoplasms is show in parentheses. (MAM-Ac, 4 hour exposures)

	Weeks Post-Exposure			
	6	13	26	48
Group I (30°C)	1/10 (1)	3/11 (3)	9/7 (8)	4/2 (4)
Group II (25°C)	0/10 (0)	5/10 (3)	4/7 (4)	3/3 (2)
Group III (20°C)	2/10 (1)	2/10 (1)	5/9 (4)	5/8 (3)

Table 3. Tissue distribution of neoplasm in Group I (30°C), Group II (25°C), and Group III (20°C) (MAM-Ac, 4 hour exposures)

		Group (# of fish with each tumor)			
Tissue	Neoplasm	I	II	III	Total
Muscle	Rhabdomyosarcoma	5	3	5	13
	Leiomyosarcoma	2	3	1	6
	Undifferentiated sarcoma	2	2	1	5
	Total Muscle Neoplasms	9	8	7	24
Liver	Hepatocellular carcinoma	2	0	0	2
	Cholangiocarcinoma	1	0	2	3
	Hepatocellular adenoma	1	2	1	4
	Cholangioma	0	1	1	2
	Total Liver Neoplasms	4	3	4	11
Swim bladder	Adenocarcinoma	2	0	0	2
	Adenoma	0	0	2	2
Peritoneum	Undifferentiated sarcoma	1	1	0	2
Gill	Hemangiosarcoma	1	0	0	1
Subcutis	Hemangioma	0	0	1	1
	Total Neoplasms All Tissues				43

In order to validate the use of aquatic bioassays to assess human health risk, the identification of lesions which may progress to neoplasia and subsequently compromise the host is mandatory. The current proposal was

designed to establish the use of immunocytochemistry on paraffin embedded fish tissues in order to begin to establish cell lineage in proliferative populations and neoplasms.

II. Body

A. Hypothesis and Significance

The majority of neoplasms, particularly well differentiated tumors, can be diagnosed by morphologic criteria. Bundles of spindle shaped cells are characteristic of sarcomas (mesenchymal origin), and glandular formation is indicative of carcinoma (epithelial origin). Within these broad groups, neoplasms can be further categorized by sets of morphologic criteria established through years of experience. However in undifferentiated tumors, often designated undifferentiated sarcoma or carcinoma, the neoplasms cannot be further classified. Even less differentiated neoplasms are sometimes given the designation "malignant tumor". Histochemical special stains have been used by pathologists to identify some features of differentiation such as the presence of certain lipids or enzymes. However, an additional powerful tool currently used in tumor diagnosis is immunohistochemistry (IHC), in which antibodies directed against certain "differentiation molecules" (substances expressed by tumor cells which indicate differentiation along a certain pathway) are used to locate these substances in tumor cells (1-3,11-13). In human pathology, IHC techniques are well developed and identification of numerous antigens in tumors is possible. The differentiation molecules include hormones (insulin, thyroglobulin, testosterone, etc.), intermediate filaments (cytokeratin, desmin, vimentin, glial fibrillary acidic proteins neurofilament proteins), so-called tumor associated antigens (antigens produced by certain tumors) and many other substances based on molecules found

in normal cell types (i.e., alpha-1-antitrypsin in liver, factor VIII in endothelial cells). By using a panel of monoclonal antibodies on a tumor, the presence and/or absence of certain antigens identifies the tumor cell lineage along a certain differentiation pathway (1-3,11-13). An example of immunodiagnosis of an undifferentiated sarcoma is shown in Figure 1 (10).

FIGURE 1. Sample immunodiagnosis of a sarcoma

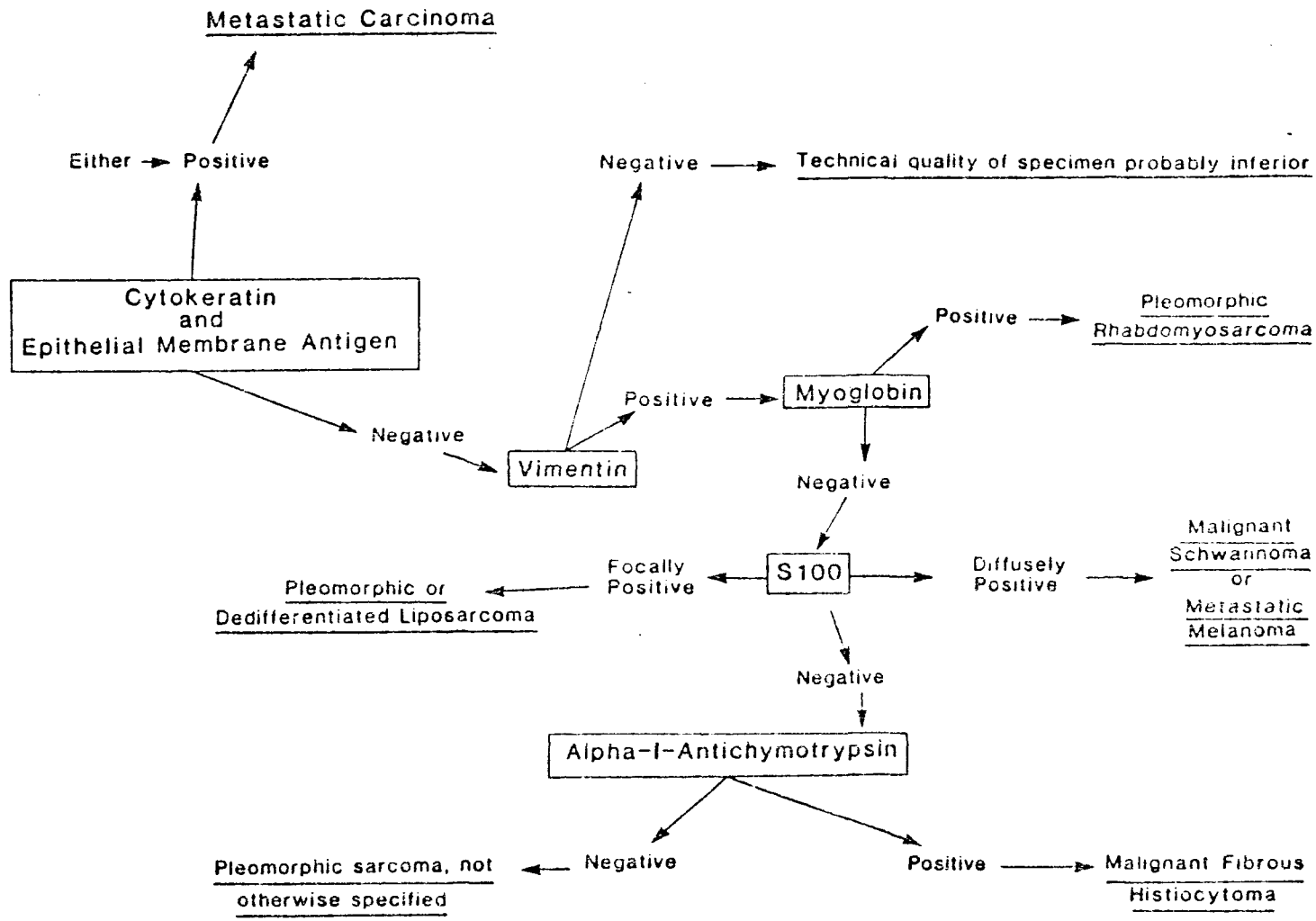


FIG. 19. Algorithm for immunodiagnosis of pleomorphic sarcomas of soft tissue.

From Wick, M.R., and Swanson, P.E., Soft Tissue Tumors. In Diagnostic Immunopathology, R.B. Colvin, A.K. Bhan, and R.T. McCluskey,eds. Raven Press, N.Y. 1988.

Immunohistochemistry must always be used in conjunction with morphology to complement a morphologic diagnosis, and not as a replacement. Generally, monoclonal antibodies produced by hybridoma cell lines are utilized in IHC rather than polyclonal antibodies due to increased antigenic specificity and decreased variability (1).

The hypothesis for this study is that the development of immunohistochemical methods for the identification of carcinogen induced neoplasms and other proliferative cellular lesions of unknown origin from paraffin embedded fish tissues will contribute significantly to the establishment of aquatic bioassays as valid, reliable alternatives in carcinogenicity and toxicity testing to assess human health risk. The development of additional diagnostic tests such as IHC are important because 1) fish tissue can respond to injury in ways atypical compared to mammals by the formation of unusual proliferative or neoplastic lesions which cannot yet be diagnosed by morphologic criteria alone, 2) these atypical lesions must be identified to determine the possible progression of these lesions to various types of neoplasms; a critical factor in determining the health effects or risks of a compound, 3) IHC methods have the unique ability to allow direct correlation of histologically detected lesions with their antigenic expression, and 4) these techniques are largely unavailable at the present time, so that this body of work is unique.

The continuation of this contract also highlights the concern of the military with environmental issues. There is enormous interest in this area of research from the scientific and public sectors as environmental pollution is finally a topic of world-wide concern. This is even more critical for field military personnel as their exposure risk to contaminated water and/or food

supplies can become considerable. The development of a small fish aquatic bioassay is important from a military standpoint because once the database is established, the aquatic bioassay 1) may provide a more rapid, less expensive alternative to rodent toxicity and carcinogenicity testing for determining military personnel health risk to noxious compounds which might be generated by military operational procedures or are present in the field from other sources, and 2) may allow determination of the effects of these potentially noxious compounds on aquatic ecosystems. These are exceedingly important goals from public, environmental, and military standpoints.

B. Methods

Two groups of 50 medaka were exposed to MAM-Ac (10 mg/L) or N-methyl-n-nitro-n-nitroso-guanidine (MNNG) (30 mg/L) at two weeks of age to induce a variety of neoplasms. Fish were sacrificed at varying ages up to 1.5 years depending on tumor development. The fish upon sacrifice were placed in Bouins fixative with small portions of liver or other grossly detectable neoplasms placed in 3.0% glutaraldehyde in 0.1M cacodylate buffer for electron microscopy.

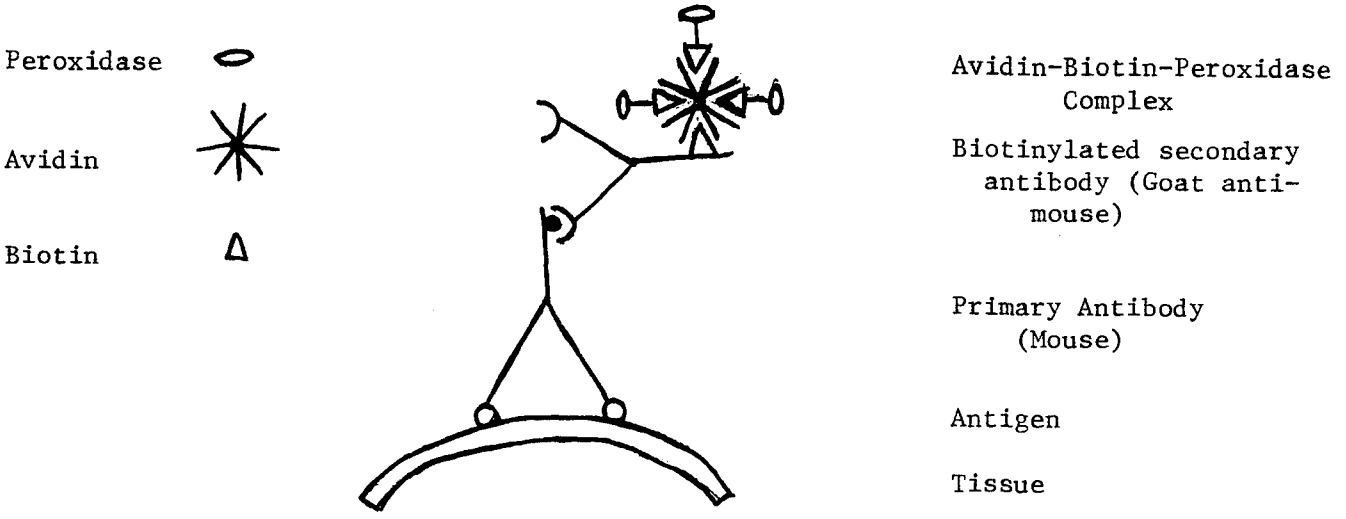
The intermediate filament proteins were chosen to develop first as they have demonstrated phylogenetic conservation in several species, and are most commonly used in tumor diagnosis (Table 4). Other antibodies tested are also shown in Table 4. The IHC method used was the strepavidin-biotin-peroxidase method (7,9) shown in Figure 2. For the preliminary studies, tissues were also taken from striped bass (Morone saxatilis) for comparison studies.

Table 4. Differentiation antigens labeled in normal tissues and neoplasms from the medaka

Antigen	Application
Intermediate filament proteins:	
*Cytokeratin (19+ different proteins 40-60KD)	Detects epithelial differentiation (carcinomas)
Vimentin (1 protein 58KD)	Detects mesenchymal differentiation (sarcomas)
Desmin (1 protein 53KD)	Detects smooth and skeletal muscle differentiation
Glial fibrillary acidic protein (GFAP - 1 protein 51 KD)	Detects glial cell differentiation
Neurofilament (3 proteins 68, 150, and 200 KD)	Detects neuronal cell origin
Other:	
Alpha-1 antitrypsin	Common marker for hepatocellular carcinoma
Factor VIII/UEA-1	Markers for endothelial cell differentiation
S-100 protein	Marker for nerve sheath tumors, melanoma
Actin	Skeletal muscle
Chromogranin	Neuroendocrine cells
Myelin associated protein	Neurons
ULEX europaeus agglutinin I	Endothelial cells

*Different cytokeratins can be used to detect different types of epithelium, i.e. to differentiate hepatocellular carcinoma from cholangiocellular carcinoma (11).

FIGURE 2. Avidin-Biotin-Peroxidase Complex Technique. Modified from A.K.Bhan, Immunoperoxidase, in Diagnostic Immunopathology



C. Results

The results of all antibodies is shown in Table 5. The majority of data is from cytokeratin antibodies studies (Tables 6, 7). In striped bass, the two cytokeratin antibodies AE1/AE3 and MAK-6 showed strong epithelial positivity in skin, gills, cornea, renal tubules, and gastrointestinal tract, simple ducts associated with pancreatic acini, bile ducts through their terminal divisions, and thymic Hassall's corpuscles. In general, Bouins fixative improved the results seen compared to formalin fixation (7).

In the medaka, skin, gills, cornea, and upper gastrointestinal tract were similarly reactive to both antibodies as seen with striped bass. However, renal tubules and bile ducts stained less intensely or not at all. A problem with adequate fixation cannot be entirely ruled out, although several methods of tissue dissection (sagittal section, transverse section, and individual organ removal) were tried in preliminary studies without significant differences in results. Another striking difference from striped bass was the poor staining of intestinal epithelium. The positive cytokeratin staining ended abruptly at the level of the intestine (the esophagus joins the intestine in the medaka), although weak, patchy staining of the intestinal epithelium was sometimes seen. This may indicate a difference between the two species of fish in the types or amounts of cytokeratins present in the intestine.

Good results have also been produced using anti-GFAP and neurofilament antibodies on fish tissues, producing typical antigenic response as seen in mammals. Anti-desmin antibodies have produced less than optimal results possibly because the protein structure is not preserved between species.

Table 5. Antibodies tested and results in medaka

Antibody against	Name	Company	Result
Cytokeratin	AE1/AE3	Boeringer Mannheim	+
	MAK-6	Triton Diagnostics	+
	CAM 5.2	Bectin Dickinson	-
Vimentin		BioGenex	+
		ICN Immunobiologics	+
Desmin		BioGenex	+/-
		DAKO	+/-
GFAP		Novacastia	-
Neurofilament		Laysystems	+
		DAKO	-
Alpha-1-antitrypsin		BioGenex	-
Factor VIII		DAKO	-
Chromogranin		Boehringer Mannheim	+
Actin		BioGenex	+
Ulex europaeus agglutinin I		Vector	-
Endothelial cell antigen		BioGenex	ND
Lysozyme		BioGenex	ND
S-100 protein		BioGenex	+
MAP-2		Boeringer Mannheim	+/-

ND=not determined to date

Table 6. Cytokeratin reactivity in striped bass relative to antibody and fixative

	Bouins Fixative		Formalin Fixative	
Tissue	AE1/AE3	MAK-6	AE1/AE3	MAK-6
Skin	+++	+++	+++	+++
Gills	+++	+++	+/++	+/++
Eye				
Cornea	+++	+++	++	+++
Retina	-	-	-	-
Liver				
Hepatocytes	-	-	-	-
Bile ducts	+++	++	+	-/+
Kidney				
Tubules	+++	++	++	++
Glomeruli	-	-	-	-
Gastrointestinal tract				
Oral cavity	+++	+++	+++	+++
Esophagus	+++	+++	+++	++
Stomach	+++	++	+++	+
Intestine	+++	++	++	+
Pancreas				
Acini	-	-	-	-
Islets	-	-	-	-
Brain/spinal cord	-	-	-	-
Spleen	-	-	-	-
Head kidney	-	-	-	-
Thymus				
Lymphocytes	-	-	-	-
Hassall's corpuscles	+++	+++	NP	NP
Muscle	-	-	-	-
Heart	-	-	-	-
Bone/cartilage	-	-	-	-
Ovary/Testis	-	-	-	-

+++ = strong; ++ = moderate; + = fair, -/+ = weak and patchy
NP = tissue not present on the slides examined

Table 7. Cytokeratin reactivity in medaka relative to antibody and fixative

	Bouins's Fixative		Formalin Fixative	
Tissue	AE1/AE3	MAK-6	AE1/AE3	MAK-6
Skin	+++	+++	++	++
Gills	+++	+++	+++	+/++
Eye				
Cornea	+++	+++	++	+++
Retina	-	-	-	-
Liver				
Hepatocytes	-	-	-	-
Bile ducts	+	-	-/+	-
Kidney				
Tubules	+	+/++	-/+	+/++
Glomeruli	-	-	-	-
Urinary bladder	+++	++	+	-/+
Gastrointestinal tract				
Oral cavity	+++	+++	+++	++
Esophagus	+++	+++	+/++	++
Intestine	-	-	-	-
Pancreas				
Acini	-	-	-	-
Islets	-	-	-	-
Brain/spinal cord	-	-	-	-
Spleen	-	-	-	-
Thymus				
Lymphoid cells	-	-	-	-
Hassalls corpuscles	+++	++	-	NP
Muscle	-	-	-	-
Heart	-	-	-	-
Bone/cartilage	-	-	-	-
Ovary/Testis	-	-	-	-
Oviduct	+	-	-	-
Thyroid	-	-	-	-

+++ = strong, ++ = moderate, + = fair, -/+ = weak and patchy
NP = tissue not present on the slides examined.

Positive results were also seen with actin (muscle), chromogranin (pituitary), and variably with MAP-2 antibodies. Negative results with other antibodies are shown in Table 5. Vimentin antibodies worked best with cryosections and stained chromatophores and periarteriolar juxtaglomerular cells, with increased staining of the stratum compactum, connective tissues around the distal esophagus, vent, and ovary, and perivascular cells in the tail musculature, gills, pancreas, and spleen with alcohol fixation.

Cytokeratin, neurofilament, and vimentin antibodies were tested on several neoplasms and proliferative lesions induced by MAM-Ac (8). Neoplasms from the liver included hepatocellular carcinoma, cholangiocarcinoma, hepatocellular adenoma, hemangiopericytoma, and hemangioma. Grossly, there was hepatomegaly or the formation of distinct nodules. The liver parenchyma in controls consisted histologically of a tubulosinusoidal distribution of hepatocytes into two plates with well defined sinusoidal and biliary borders. Endothelial cells lining sinusoids were identified primarily by their small dense oval nuclei. Larger biliary ductules consisted of cuboidal cells with large oval nuclei with dispersed chromatin, while smaller branches of the biliary tree, and perisinusoidal cells, were largely inapparent. Biliary epithelial cells reacted positively for cytokeratin, while hepatocyte, endothelial cells, and peribiliary connective tissue cells were negative. The hepatocellular carcinomas were all moderately to well differentiated, ranging in appearance from micronodules of disorganized cells separated by spindle cell trabeculae, to areas of evenly distributed trabeculae. It was not uncommon to see marked biliary hyperplasia in association with hepatocellular carcinoma. A few neoplasms consisted entirely or in part of markedly distended cells with abundant pale foamy cytoplasm and enlarged nuclei (megalocytes). Ultrastructurally in well differentiated neoplasms, hepatocellular features were present such as round nuclei with prominent

nucleoli, prominent strands of round endoplasmic reticulum often surrounding mitochondria, peroxisomes, and canaliculi. However, foci of cells with a higher nucleus to cytoplasmic ratio and lack of peroxisomes and other distinguishing hepatocellular features were also seen. The hepatocellular carcinomas and adenoma stained negatively for cytokeratin. The cholangiocarcinomas were variable in appearance, with more differentiated neoplasms consisting of ductules with evenly distributed cuboidal to columnar cell linings. Less differentiated neoplasms had bizarre, distorted arrangements of cells with markedly variable acini or formed nearly solid sheets of cells with inapparent acini. There was metastasis of cholangiocarcinoma to the spleen and kidney in one fish, which was the only metastasis in the study. All cholangiocarcinomas were positive for cytokeratin to variable degrees, with the more differentiated neoplasms showing strong reactivity. The hemangiopericytoma was identical to what has been previously described (6), consisting of whorls and sheets of spindle shaped cells with small oval dense nuclei. Both this neoplasm and the small hemangioma were negative for cytokeratin.

There were also several extrahepatic neoplasms located primarily within the peritoneum. Three neoplasms were characterized by shaggy irregular proliferations of round plump cells on frond-like connective tissue septae which covered the serosal surfaces of the intestines, ovary, and other peritoneal structures. Solid, more fibrous areas were also present, with occasional epithelium-lined clefts. All three were positive for cytokeratin, and were diagnosed as mesothelioma based on pattern of growth and cellular morphology. A pancreatic acinar cell carcinoma in peritoneal fat was characterized by a grossly detectable mass of closely associated small cellular acini with amphophilic cytoplasm and a high mitotic rate. The cells appeared well differentiated towards pancreatic acini and were negative for

cytokeratin.

In many cases there were also proliferative populations of cells which often assumed a spindloid appearance. Based on variable nuclear morphology, the lesions appeared to be comprised of mixed populations, but specific cell types were often difficult to determine histologically. Hyperplastic biliary epithelial cells, which were commonly present and reacted positively to cytokeratin antibodies, could sometimes be identified by their oval vesicular nuclei, and formation of ductules. However, a relatively strong biliary component could be obscured in lesions were macrophages were abundant and/or when ductular lumens, readily identified ultrastructurally were inapparent histologically. Therefore, an increased level of biliary hyperplasia was identified by immunohistochemistry or electron microscopy than was appreciated by histology alone.

Another characteristic lesion consisted of loosely arranged spindle cells with long interconnecting cytoplasmic processes compatible with spongiosis hepatic. Large areas of liver were sometimes involved. The cells forming these lesions had convoluted nuclei, often prominent desmosomal attachments, bundles of cytoplasmic filaments, and no "lumenal" morphology such as microvilli seen with biliary epithelium. Macrophages were abundant in the spongiosis lesions, and along with spongiosis cells reacted negatively to cytokeratin antibodies. A less common lesion was characterized by thick bands or circular swirls of spindle cells involving a minimal to moderate amount of parenchyma.

III. Conclusions

The results indicate good phylogenetic conservation of intermediate filament and other protein antigens. These data can be widely applied in fish carcinogenesis research to continue to assess cells lineage and thus determine the roles of certain cells in neoplastic progression. The next step is to

confirm the presence of intermediate filament proteins in tissues and neoplasms through isolation and characterization studies, and to continue to assess the various antibodies on fish tissues. In rodent carcinogenesis (mouse papilloma virus model), it is also possible to predict the progression of neoplasms by identifying certain types of intermediate filaments. It would be interesting to determine if this is also true with fish neoplasms.

The streptavidin-HRP method used is simple, direct, and produced clear, reproducible results. Although investigators will need to develop independent standards for each species of fish, the broad cross reactivity seen between these two divergent species of fish and the mammalian controls is indicative of the potential for use of this technique as a diagnostic tool in fish carcinogenesis research.

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APPENDIX 1

ABSTRACTS:

Bunton TE, Wolfe MJ, Gardner HS, Toussaint MW: Light microscopic and immunocytochemical evaluation of unusual neoplasms induced in medaka (Oryzias latipes) by N-methyl,N'-nitro-N-nitrosoguanidine. American Fisheries Society Meeting, Seattle, WA, September, 1994.

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